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N-Linked Glycosylation Facilitates Sialic Acid-Independent Attachment and Entry of Influenza A Viruses into Cells Expressing DC-SIGN or L-SIGN▽

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It is widely recognized that sialic acid (SA) can mediate attachment of influenza virus to the cell surface, and yet the specific receptors that mediate virus entry are not known. For many viruses, a definitive demonstration of receptor function has been achieved when nonpermissive cells are rendered susceptible to infection following transfection of the gene encoding a putative receptor. For influenza virus, such approaches have been confounded by the abundance of SA on mammalian cells so that it has been difficult to identify cell lines that are not susceptible to infection. We examined influenza virus infection of Lec2 Chinese hamster ovary (CHO) cells, a mutant cell line deficient in SA. Lec2 CHO cells were resistant to influenza virus infection, and stable cell lines expressing either DC-SIGN or L-SIGN were generated to assess the potential of each molecule to function as SA-independent receptors for influenza A viruses. Virus strain BJx109 (H3N2) bound to Lec2 CHO cells expressing DC-SIGN or L-SIGN in a Ca^{2+}-dependent manner, and transfected cells were susceptible to virus infection. Treatment of Lec2-DC-SIGN and Lec2-L-SIGN cells with mannan, but not bacterial neuraminidase, blocked infection, a finding consistent with SA-independent virus attachment and entry. Moreover, virus strain PR8 (H1N1) bears low levels of mannose-rich glycans and was inefficient at infecting Lec2 CHO cells expressing either DC-SIGN or L-SIGN, whereas other glycosylated H1N1 subtype viruses could infect cells efficiently. Together, these data indicate that human C-type lectins (DC-SIGN and L-SIGN) can mediate attachment and entry of influenza viruses independently of cell surface SA.

Attachment of influenza A virus to sialic acid (SA) on the cell surface is a critical first step in the initiation of infection (56). More specifically, the receptor-binding site (RBS) of the viral hemagglutinin (HA) glycoprotein binds to SA expressed by cell surface glycoproteins and/or glycolipids to mediate virus attachment. On mammalian cells, SA generally forms glycosidic linkages with the underlying galactose (Gal) residues in SA-(α-2,3)-Gal or SA-(α-2,6)-Gal configurations (56), and this is a critical factor in determining the tropism of influenza virus for particular host cells (53, 54). SA-(α-2,3)-Gal is expressed throughout the avian gastrointestinal tract and is preferentially bound by avian influenza A viruses (67), whereas SA-(α-2,6)-Gal is abundant in the human respiratory tract and is the preferred linkage recognized by human virus strains (70).

Despite the important role of HA-mediated recognition of SA, SA-independent entry of influenza virus into host cells has been reported (64). Moreover, the availability of SA on the cell surface does not always result in productive infection (33). Of interest, Chu and Whittaker reported that Lec1 cells, a mutant Chinese hamster ovary (CHO) cell line deficient in expression of N-linked glycans (44, 61), were resistant to influenza virus infection, despite retaining full capacity for virus binding and fusion and having no defect in their inherent ability to support viral replication (12). Hence, despite an abundance of cell surface SA, Lec1 cells appeared to lack the specific receptor(s) required for endocytosis and internalization of virions. Thus, binding to SA facilitates attachment of influenza virus to the cell surface; however, the specific receptors that mediate virus entry have not been identified.

We have previously investigated the role of Ca^{2+}-dependent (C-type) lectins in mediating infectious entry of influenza virus into murine macrophages (Mϕ) (49, 73). In these studies, influenza virus was shown to bind to the Mϕ mannose receptor (MMR) by SA-dependent and SA-independent mechanisms, whereas recognition of virus by the macrophage galactose-like lectin (MGL) was independent of SA and occurred by Ca^{2+}-dependent recognition of glycans on the HA and/or neuraminidase (NA) glycoproteins of the virus. Moreover, multivalent ligands of MMR and MGL inhibited influenza virus infection in a manner that correlated with expression of each receptor on different Mϕ populations. These studies are informative but indirect and do not elucidate the specific role of C-type lectins in attachment and/or entry of influenza virus into murine Mϕ.

For many viruses, identification of cell surface receptors has been demonstrated following the transfection of gene(s) encoding putative receptor(s) into a cell line that is resistant to infection, such that the cells are rendered susceptible to virus entry. Such approaches have been utilized to identify functional receptors for herpes simplex virus (41) and reovirus (3) and to identify a coreceptor for HIV-1 (22). In the case of

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influenza virus, such approaches are confounded by the abundance of SA on the surface of mammalian cells such that it has been difficult to identify cell lines that are not susceptible to at least the early stages of virus infection. In the present study, we demonstrate that Lec2 CHO cells, a mutant cell line deficient in terminal SA residues due to a defect in transport of SA across Golgi vesicles by the CMP-SA transporter (44, 63), are resistant to influenza virus infection. Furthermore, we have used Lec2 CHO cells to develop a transfection-based approach to investigate SA-independent interactions between influenza virus and two human Ca^{2+}-dependent (C-type) lectins.

DC-SIGN (CD209) and L-SIGN (DC-SIGNR and CD209L) are closely related C-type lectins that recognize a variety of microbes, including viruses (32, 59). Both are tetrameric type II transmembrane proteins that contain C-type carbohydrate recognition domains that interact with mannose-rich oligosaccharides (21, 40). DC-SIGN is expressed at high levels by monocyte- and $CD34^+$-derived subsets of immature and mature dendritic cells (DCs) (24, 25), as well as alveolar Mϕ (60). The expression pattern of L-SIGN is very different from that of DC-SIGN, being restricted predominantly to endothelial cells, including those in the lung and lymph nodes, as well as lung alveolar epithelial cells (20, 30). Previous studies have demonstrated that DC-SIGN and/or L-SIGN act as capture and/or entry receptors for a number of enveloped viruses, including HIV-1 (21, 24, 58, 72), SARS-CoV (29, 39), West Nile virus (WNV [16]), hepatitis C virus (38), Ebola virus (37), and dengue virus (69). Recently, DC-SIGN has shown to act as an attachment receptor for H5N1 influenza virus, enhancing virus infection in *trans*, as well as promoting virus entry in *cis* via secondary interactions with sialylated cell surface molecules on the same cell (76).

We generated stable Lec2-transfected cell lines expressing either human DC-SIGN or L-SIGN at the cell surface and show that both receptors can mediate attachment and entry of influenza virus strain BJx109 (H3N2). Treatment of transfected CHO Lec2 cells with mannan, but not bacterial neuraminidase (sialidase), blocked infection, a finding consistent with SA-independent infection via C-type lectins. Finally, we demonstrate that virus bearing low levels of mannose-rich glycans (PR8 [H1N1]) was inefficient at infecting Lec2 cells expressing either DC-SIGN or L-SIGN. Together, these data demonstrate that DC-SIGN and L-SIGN can recognize mannose-rich glycans on influenza virus to mediate SA-independent attachment and infection of cells.

MATERIALS AND METHODS

Cell lines. CHO Pro-5 cells (62) were obtained from the American Type Culture Collection (ATCC), Manassas, VA. The glycosylation mutant cell line, Lec2, derived from CHO Pro-5 cells (19, 44, 63) was also obtained from the ATCC. Both cell lines were cultured in alpha-minimal essential medium (αMEM; Gibco-BRL, New York) supplemented with 10% (vol/vol) fetal calf serum (JRH Biosciences, Kansas), 4 mM L-glutamine, 100 IU of penicillin, 10 μg of streptomycin/ml, nonessential amino acids (Gibco-BRL), and 50 μM β-mercaptoethanol. LA-4 cells were also obtained from the ATCC and cultured in Kaighn's modification of Ham F-12 medium (Gibco), supplemented as described above.

Generation of Lec2 cells transfected with DC-SIGN and L-SIGN. Transfected Lec2 cells were generated by using neomycin-resistant plasmids encoding human DC-SIGN (46) or human L-SIGN (47). The pcDNA3-DC-SIGN plasmid was obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Disease (NIAID), National Institutes of Health (NIH). pcDNA3-DC-SIGN was obtained from S. Pohlmann, F. Baribaud, F. Kirchhoff, and R. W. Doms, and the pcDNA3-L-SIGN plasmid was obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH. pcDNA3-DC-SIGNR was obtained from S. Pohlman, E. Soilleux, F. Baribaud, and R. W. Doms. Subconfluent Lec2 cells in six-well tissue culture plates (Nunc, New York) were transfected by using FuGene 6 transfection reagent (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. Control-transfected Lec2 cells (designated Lec2-control cells) were generated by using a neomycin-resistant plasmid expressing cytoplasmic hen egg ovalbumin (OVA) (6) generously donated by A. M. Lew and J. L. Brady, The Walter and Eliza Hall Institute, Parkville, Australia. This construct lacks the sequence for cell surface trafficking ensuring intracellular expression of the OVA protein. For the selection of stable transfectants expressing DC-SIGN and L-SIGN or cytoplasmic OVA, transfected cells were cultured in the presence of 1 mg of the selective antibiotic G418 (Gibco)/ml. Clones with high surface expression of DC-SIGN and L-SIGN were obtained via limiting dilution and selected by flow cytometric analysis after staining with monoclonal antibodies (MAbs) directed to human DC-SIGN (clone 120507, conjugated to allophycocyanin [APC]; R&D Systems, Inc.) or human L-SIGN-PE (clone 120604, conjugated to phycoerythrin [PE]; R&D Systems, Inc.). Lec2-DC-SIGN, Lec2-L-SIGN, and Lec2-OVA cells were cultured in supplemented αMEM as described above, in the presence of 1 mg of G418/ml to maintain transgene expression.

Viruses. The influenza A virus strains used in the present study were A/PR/8/34 (PR8, H1N1) and BJx109 (H3N2), a high-yielding reassortant of PR8 with A/Beijing/353/89 (Beij/89; H3N2) bearing the H3N2 surface glycoproteins. The seasonal H1N1 virus strains A/New Caledonia/20/1999 (New Cal/99) and A/Solomon Islands/3/2006 (Sol Is/06) were obtained from the World Heath Organization Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia. Based on sequence analysis, New Cal/99 and Sol Is/06 both bear four potential sites of N-linked glycosylation on the head of HA (GenBank accession numbers AY289929 and CY031340, respectively). All viruses were grown in 10-day-old embryonated eggs by standard procedures and titrated on Madin-Darby canine kidney (MDCK) cells, and viruses were purified from allantoic fluid by rate zonal sedimentation on 25 to 75% (wt/vol) sucrose gradients, as described previously (2).

Additional reassortant influenza viruses used in the present study were generated by eight-plasmid reverse genetics as previously described (42). The viruses included (i) 7:1 reassortants consisting of the PR8 backbone with either the HA or NA gene from Beij/89 (RG-PR8-Beij/89 HA and RG-PR8-Beij/89 NA, respectively), (ii) eight genes from PR8 (RG-PR8), and (iii) a 6:2 reassortant consisting of six genes from PR8 and the HA and NA from Beij/89 (RG-PR8-Beij/89 HA/NA). The rescued viruses were recovered after 3 days and amplified in the allantoic cavity of 10-day-old embryonated hens' eggs.

Binding of lectin, influenza virus, and mannan to the cell surface. Levels of cell surface SA-(α-2,3)-Gal were determined by using the biotinylated plant lectin *Maackia amurensis* agglutinin II (MAA; EY Laboratories, California), which binds specifically to SA-(α-2,3)-Gal. Cells were detached from plastic flasks using 0.75 mM EDTA in Tris-buffered saline (TBS; 0.05 M Tris-HCl in 0.15 M NaCl [pH 7.4]) and incubated with 5 μg of biotinylated-MAA (b-MAA)/ml in lectin buffer (TBS containing 10 mM $CaCl_2$ and 1 mg of bovine serum albumin [BSA]/ml; Sigma-Aldrich, Missouri) at 4°C for 30 min. The cells were washed, and bound MAA was detected by using streptavidin conjugated to APC and flow cytometry. To ensure binding specificity, cells were pretreated for 30 min at 37°C with 200 mU of broad-spectrum bacterial sialidase derived from *Vibrio cholerae* (type III; Sigma Aldrich)/ml to remove SA prior to b-MAA binding.

The ability of influenza virus to bind to cells was determined by using a virus-binding assay. Briefly, detached cells were incubated with 5 μg of purified BJx109 virus/ml in lectin buffer at 4°C for 30 min. Bound virus was detected by using biotinylated MAb recognizing the HA of Beij/89 (MAb C1/1; L. E. Brown, produced in the Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Australia) in conjunction with streptavidin conjugated to APC and flow cytometry. All antibody-binding steps were performed at 4°C in lectin-binding buffer. In experiments assessing the Ca^{2+} dependence of virus binding, $CaCl_2$ was omitted from the lectin-binding buffer and replaced with 10 mM EDTA.

Binding of mannan to the cell surface was assessed by using an adaptation of the virus-binding assay. Briefly, detached cells were incubated with biotin-labeled mannan (b-mannan; 10 μg/ml) in lectin-binding buffer for 1 h at 4°C. Biotinylated mannan used in the study was prepared as previously described (31). Bound mannan was detected using streptavidin conjugated to APC in conjunction with flow cytometry. In experiments assessing the Ca^{2+} dependence of mannan bind-

ing, $CaCl_2$ was omitted from the lectin-binding buffer and replaced with 10 mM EDTA.

Virus infection assays. Cells were cultured overnight in eight-well chamber slides (Lab-Tek), and confluent cell monolayers were washed with serum-free αMEM and infected with influenza virus as described previously (49). At 6 to 8 h postinfection, slides were washed in phosphate-buffered saline (PBS), fixed in 80% (vol/vol) acetone, and stained with MAb MP3.10g2.IC7 (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia), specific for the nucleoprotein (NP) of type A influenza viruses, followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark). The percentage of infected cells was determined by costaining with propidium iodide and counting the total number of cells versus FITC-positive cells under ×100 magnification. A minimum of four random fields were selected for counting, assessing at least 200 cells for each sample. Infected cells were photographed by using a Leica DMLB microscope (Leica Microsystems, Germany) and a Leica DFC 490 camera in conjunction with Leica IM50 Image Manager software. In some experiments, cell monolayers were pretreated before infection with 200 mU of bacterial sialidase from *Vibrio cholerae* type III (Sigma-Aldrich)/ml, *Clostridium perfringens* (Sigma-Aldrich) or *Arthrobacter ureafaciens* (Roche), or with 10 mg of mannan (Sigma-Aldrich)/ml in serum-free medium for 1 h at 37°C to remove cell surface SA or block C-type lectins, respectively.

To determine release of infectious virus from cells, confluent cell monolayers were infected in chamber slides as described above. Cell supernatants were collected at 2 and 24 h postinfection, incubated with TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (10 μg/ml; Sigma-Aldrich) for 45 min at 37°C to facilitate cleavage of the viral HA_0 (56), and titers of infectious virus were determined by plaque assay on MDCK cells.

qRT-PCR for influenza mRNA and vRNA. Viral RNA (vRNA) and mRNA in virus-infected cells were determined by using quantitative real-time reverse transcription-PCR (qRT-PCR). Briefly, 10^6 cells in 24-well tissue culture plates were infected with 2×10^7 PFU of BJx109 for 1 h at 37°C, washed twice, and incubated in serum-free medium. At 2 and 8 h postinfection, RNA was extracted from cells by using an RNeasy minikit (Qiagen) and stored at −70°C. Levels of matrix (M) gene vRNA and mRNA were determined via qRT-PCR using TaqMan chemistry. The primers and probes for the influenza A virus M gene were as follows: forward primer, 5′-GAC CRA TCC TGT CAC CTC TGA C-3′; reverse primer, 5′-GGG CAT TYT GGA CAA AKC GTC TAC G-3′; and probe, 5′-FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1 (17). The forward primer was used for vRNA detection, and the reverse primer was used for mRNA detection with the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) to produce cDNA. cDNA was then used for real-time PCR with TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 fast real-time PCR instrument (Applied Biosystems) using a standard program. vRNA and mRNA copy numbers were calculated according to a standard curve generated using 10^1, 10^2, 10^3, 10^4, and 10^6 copies of M gene RNA.

ELISA for binding of concanavalin A to influenza virus. Enzyme-linked immunosorbent assay (ELISA) plates were coated overnight with 50 μl of purified influenza virus in PBS and then blocked for 1 h with BSA (10 mg/ml). The wells were washed with PBS containing 0.05% Tween 20 (PBST) and incubated for 2 h with 2 mg of biotin-labeled concanavalin A (ConA; Sigma-Aldrich)/ml in PBS containing 5 mg of BSA per ml. The wells were washed again, and bound lectin was detected after incubation with streptavidin conjugated to horseradish peroxidase (HRP; Silenus, Victoria, Australia). To confirm that different viruses bound to ELISA wells with similar efficiency, additional virus-coated and blocked wells were incubated for 2 h with MAb 165, which recognizes the cross-reactive host antigen common to all egg-grown influenza viruses (50), followed by sheep anti-mouse immunoglobulins conjugated to HRP (Silenus).

Western blotting and virus overlay protein blot assays (VOPBAs). Whole-cell lysates were prepared using a buffer comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (vol/vol) Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and broad-spectrum protease inhibitor cocktail (Roche, Manheim, Germany), followed by incubation with cells for 1 h on ice. Lysates were clarified by centrifugation at $10{,}000 \times g$ for 3 min. Protein concentrations were determined by Bradford assay (Bio-Rad Protein Dye; Bio-Rad, California). Samples (~10 μg of protein) were boiled for 5 min before separation by SDS-PAGE under nonreducing conditions using 10 to 12.5% gels, followed by transfer to polyvinylidene difluoride (PVDF) membrane (Millipore) in Tris-glycine transfer buffer (25 mM Tris containing 192 mM glycine and 10% [vol/vol] methanol; pH 8.3).

To detect DC-SIGN and L-SIGN in whole-cell lysates by Western blot, membranes were blocked in phosphate-buffered saline (PBS) containing 0.5% (wt/vol) BSA and 0.1% (vol/vol) Tween 20 overnight at 4°C after protein transfer. All subsequent wash and antibody binding steps were performed in PBS containing 0.05% (vol/vol) Tween 20. Membranes were then incubated for 1 h at room temperature with an MAb recognizing both DC- and L-SIGN (clone DC28; R&D Systems, Inc.) that had been labeled with biotin (EZ-link Sulfo-NHS-LC-LC-Biotin; Pierce, Illinois) according to the manufacturer's instructions. Bound MAb was detected using SA-HRP in conjunction with enhanced chemiluminescence (ECL; Western Lightning Plus ECL; Perkin-Elmer, Connecticut). Blots were developed by using the Kodak Image Station 4000Mm, and images were managed by using Adobe Photoshop software.

To detect virus binding, VOPBAs were performed where membranes were blocked with VOPBA buffer (TBS containing 1% [wt/vol] BSA, 0.05% [vol/vol] Tween 20, and 5 mM $CaCl_2$) for 3 to 4 h at 4°C. Purified BJx109 (2 μg/ml) was incubated overnight, the membrane was washed, and 2 μg of biotinylated MAb C1/1 was added/ml. Bound virus was detected by using streptavidin-conjugated HRP and ECL imaging, as described above. Note that all steps were performed in VOPBA buffer and that antibody binding was performed at 4°C. To assess the Ca^{2+} dependence of virus binding, $CaCl_2$ was omitted from the VOPBA buffer and replaced with 10 mM EDTA.

Statistical analysis. Graphing and statistical analysis of data was performed by using GraphPad Prism (GraphPad Software, San Diego, CA). For comparison of multiple data sets, a one-way analysis of variance (ANOVA) with Tukey's multiple comparative analysis was used. A P value of ≤ 0.05 was considered significant.

RESULTS

Lec2 cells are deficient in cell surface SA. Lec2 cells, a mutant CHO cell line, are deficient in terminal SA residues due to a defect in the transport of SA across Golgi vesicles arising from lack of functional CMP-SA transporter (44, 63). Parental CHO cells express SA-(α-2,3)-Gal, but not SA-(α-2,6)-Gal, due to a deficiency in galactoside α-2,6-sialyltransferase (14, 35). Therefore, we used the plant lectin MAA to detect SA-(α-2,3)-Gal on parental CHO Pro5 cells and on Lec2 CHO cells. CHO Pro5 cells bound high levels of MAA, and binding to Lec2 cells was markedly reduced (Fig. 1A). As expected, desialylation of CHO Pro5 cells with bacterial sialidase led to a marked reduction in MAA binding. Desialylation of the Lec2 cells was also associated with a modest reduction in b-MAA binding (Fig. 1A, upper right panel), which is consistent with studies demonstrating that levels of SA on Lec2 cells are drastically reduced, but not absent, compared to CHO Pro5 cells (36, 63). Lec2 cells were grown in media containing fetal bovine serum, a potential source of gangliosides to cells (23, 34), which may explain the modest binding of b-MAA to the cell surface.

Influenza A virus is poor in its ability to bind to or infect Lec2 cells. As numerous studies have demonstrated the importance of cell surface SA in mediating influenza virus infection (reviewed by Skehel and Wiley [56]), we investigated the ability of influenza virus to bind to and infect SA-deficient Lec2 cells. Consistent with previous reports (12), influenza virus (strain BJx109) bound efficiently to the surface of CHO Pro5 cells (Fig. 1B), and the cells were susceptible to infection (Fig. 1C). In contrast, BJx109 bound poorly to Lec2 cells (Fig. 1B), and the fluorescence intensity was similar to that of the mock control (i.e., CHO Pro5 cells with no virus added). Moreover, Lec2 cells were largely resistant to infection by BJx109 (6% ± 2% compared to 59% ± 5% infected cells for Lec2 and CHO Pro5 cells, respectively, at the highest inoculum dose tested). The differential ability of BJx109 to infect Lec2 and CHO Pro5 cells was confirmed by using a fluorescence-activated cell sorting (FACS)-based approach where 70% ± 9% of CHO cells expressed intracellular NP at 8 h postinfection compared to 4% ± 2% of Lec2 cells using an inoculum dose of 50 PFU per

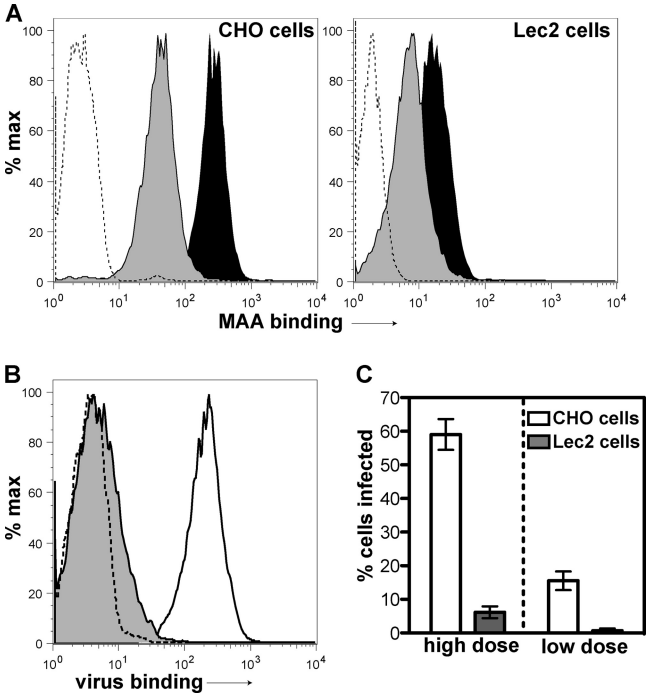

FIG. 1. Lec2 cells are deficient in cell surface SA, do not bind influenza virus, and are largely resistant to influenza virus infection. (A) FACS histograms showing SA-(α-2,3)-Gal expression on parental cells (CHO Pro-5) compared to Lec2 CHO cells. Cells were incubated in either media alone (mock, black histograms), or in media supplemented with 200 mU of bacterial sialidase/ml (gray histograms) and, after washing, the binding of b-MAA was determined. Dashed lines represent Pro5 or Lec2 cells stained with streptavidin-APC conjugate alone. (B) The binding of influenza virus BJx109 to CHO Pro5 cells (white histogram) and Lec2 CHO cells (gray histogram) was determined by flow cytometry, as described in Materials and Methods. The dashed line represents CHO Pro5 cells stained with streptavidin-APC conjugate alone. (C) Influenza virus does not infect Lec2 CHO cells efficiently. Monolayers of CHO Pro5 cells (CHO cells) or Lec2 CHO cells (Lec2 cells) were incubated with 10^7 or 10^6 PFU (indicated as high or low dose, respectively) of BJx109 for 1 h at 37°C. Monolayers were washed, incubated 6 to 8 h and then fixed and stained by immunofluorescence for expression of newly synthesized viral NP. The data represent the mean percent infection (± the SEM from a minimum of four independent fields per chamber and are representative of at least three independent experiments.

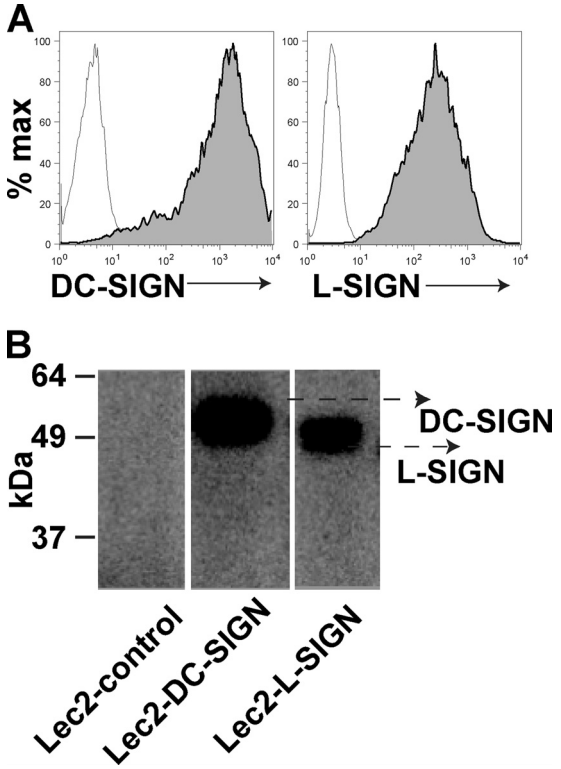

FIG. 2. Total and cell surface expression of DC-SIGN and L-SIGN by Lec2 CHO cell transfectants. (A) FACS histograms showing expression of human C-type lectins on the surface of transfected Lec2 cells (gray histograms). White histograms represent control-transfected Lec2 cells expressing intracellular OVA and stained for DC-SIGN/L-SIGN, as indicated. (B) Western blot analysis showing expression and migration pattern of DC-SIGN and L-SIGN in lysates from transfected Lec2 cells. Cell lysates from Lec2-OVA (Lec2-control), Lec2-DC-SIGN, or Lec2-L-SIGN cells were resolved by SDS-PAGE under nonreducing conditions and transferred to a PVDF membrane. To detect human C-type lectins, the blot was incubated with MAb DC28, which is cross-reactive with both DC-SIGN and L-SIGN, and developed as described in Materials and Methods. Arrows indicate bands corresponding to the approximate molecular masses of DC-SIGN and L-SIGN (ca. 49 to 55 kDa). The data are representative of at least three independent experiments.

cell (data not shown; values represent means ± the standard errors of the mean [SEM] from three independent experiments).

DC-SIGN and L-SIGN are expressed on the surface of Lec2 CHO cells after transfection. To examine the potential of human C-type lectins to act as attachment and/or entry receptors for influenza A virus, we transfected Lec2 CHO cells with human DC-SIGN and L-SIGN and selected for clones showing stable cell surface expression of each receptor as described in Materials and Methods. Flow cytometric analyses confirmed high levels of cell surface expression of either DC-SIGN (Fig. 2A, left panel) or L-SIGN (Fig. 2A, right panel) on Lec2-DC-SIGN and Lec2-L-SIGN cells, respectively, compared to control cells expressing intracellular OVA (Lec2-control).

To confirm the expression of proteins of appropriate molecular size and establish the migration patterns of DC-SIGN and L-SIGN proteins expressed by Lec2 cells, Western blot analysis of total cell lysates prepared from Lec2-control, Lec2-DC-SIGN, or Lec2-L-SIGN cells was performed using a cross-reactive MAb that detects both DC-SIGN and L-SIGN. As seen in Fig. 2B, proteins 49 to 55 kDa in size were detected in lysates from Lec2-DC-SIGN and Lec2-L-SIGN cells but were absent in Lec2-control cells.

DC-SIGN and L-SIGN expressed on Lec2 cells retain Ca^{2+}-dependent lectin-binding function. DC-SIGN and L-SIGN contain C-type carbohydrate recognition domains that bind to mannose-rich oligosaccharides (21, 40). Lec2 CHO cells are deficient in terminal SA and, based upon the published sequences, DC-SIGN (GenBank accession number M98457) and L-SIGN (GenBank accession number AF245219) are predicted to have one site of N-linked glycosylation that may be sialylated. Since the expression of MMR, a related C-type lectin, in Lec2 cells was associated with major defects in lectin binding and internalization (65), it was critical to determine

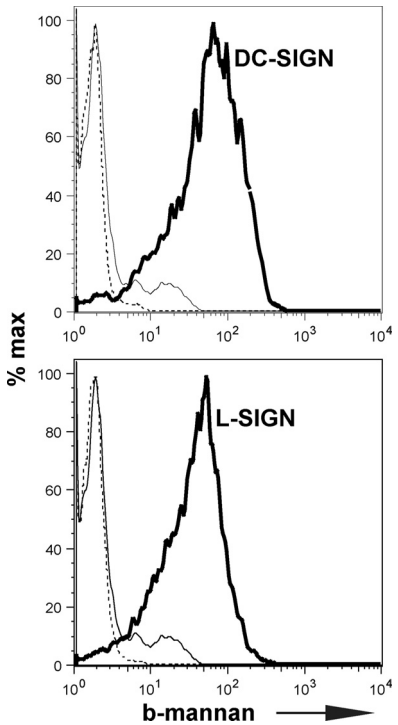

FIG. 3. DC-SIGN and L-SIGN expressed on the surface of Lec2 cells retain Ca^{2+}-dependent lectin activity. Lec2 cells expressing intracellular OVA (thin black line) or cell surface DC-SIGN or L-SIGN (thick black line in upper and lower panels, respectively) were incubated with b-mannan at 4°C for 30 min in the presence of 5 mM $CaCl_2$. After washing, cell surface b-mannan was detected by using streptavidin-APC. Lec2-DC-SIGN and Lec2-L-SIGN cells were also incubated with b-mannan in the presence of 10 mM EDTA, and the binding of b-mannan was determined (dashed line). The data are representative of at least two independent experiments.

whether DC-SIGN and L-SIGN expressed on the surface of Lec2 cells retained functional integrity by assessing their ability to bind mannan, a complex plant polysaccharide rich in terminally branched mannose residues. Flow cytometric analysis demonstrated that both Lec2-DC-SIGN (Fig. 3, upper panel) and Lec2 L-SIGN (lower panel), but not Lec2-control cells, bound mannan. Moreover, the presence of EDTA abrogated binding of mannan to Lec2 cells transfected with either DC-SIGN or L-SIGN. Together, these data demonstrate that DC-SIGN and L-SIGN expressed on the surface of Lec2 CHO cells retain C-type lectin activity.

Influenza virus binds to Lec2-DC-SIGN and Lec2-L-SIGN cells in a Ca^{2+}-dependent manner. Given that DC-SIGN and L-SIGN expressed on Lec2 cells retained C-type lectin activity, we next assessed the ability of influenza virus to bind to the surface of Lec2-DC-SIGN and Lec2-L-SIGN cells. As seen in Fig. 4A, BJx109 virus bound to Lec2-DC-SIGN (upper panel) and Lec2-L-SIGN (lower panel) cells but not to Lec2-control cells, and binding was abolished in the presence of EDTA. Binding of influenza virus to DC-SIGN and L-SIGN was also examined by VOPBA, wherein cell lysates of Lec2-DC-SIGN, Lec2-L-SIGN, or Lec2-control cells were separated by SDS-PAGE and probed with BJx109 in the presence of Ca^{2+} (Fig. 4B, left panel) or EDTA (Fig. 4B, right panel). Detection of

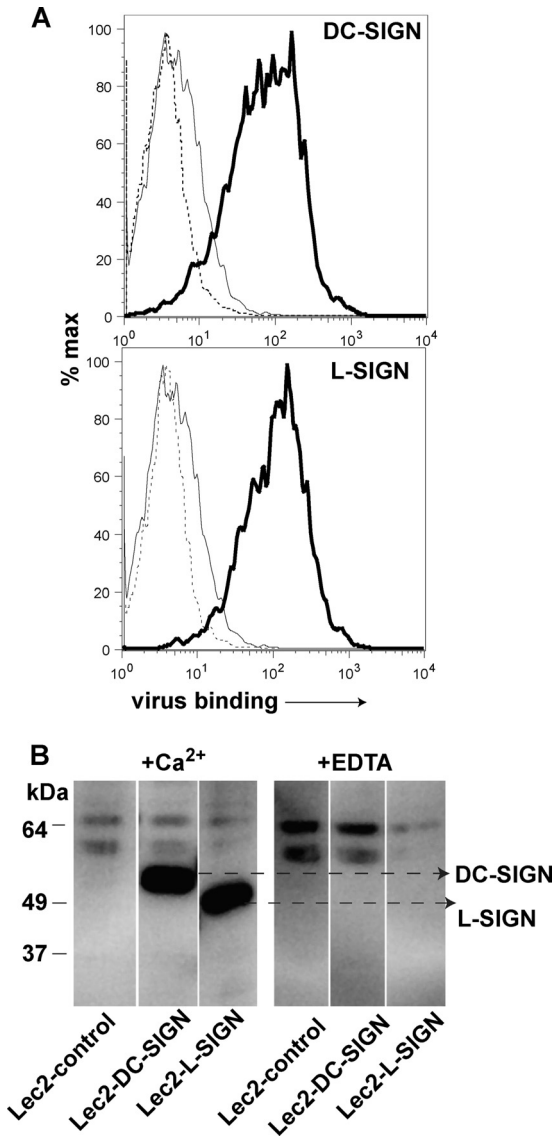

FIG. 4. DC-SIGN and L-SIGN expressed by Lec2 CHO cells mediates Ca^{2+}-dependent binding to influenza virus. (A) Binding of influenza virus BJx109 to Lec2-control (thin black lines), Lec2-DC-SIGN (thick black line, upper panel), or Lec2-L-SIGN cells (thick black line, lower panel) was determined at 4°C in the presence of 5 mM $CaCl_2$. Binding of BJx109 to Lec2-DC-SIGN or Lec2-L-SIGN cells was also determined at 4°C in the presence of 10 mM EDTA (dashed lines). The data are representative of at least two independent experiments. (B) VOPBA demonstrating Ca^{2+}-dependent binding of influenza virus BJx109 to proteins corresponding to DC-SIGN and L-SIGN. Whole-cell lysates from Lec2-control, Lec2-DC-SIGN, or Lec2-L-SIGN cells were electrophoresed by SDS-PAGE under nonreducing conditions, transferred to PVDF membrane, and probed with influenza virus BJx109 in the presence of either 5 mM $CaCl_2$ (Ca^{2+}) or 10 mM EDTA. Arrows indicate bands corresponding to the approximate molecular masses of DC-SIGN and L-SIGN (ca. 49 to 55 kDa). The data are representative of two independent experiments.

bound virus revealed interactions with numerous proteins, with particularly intense binding to species of 49 to 55 kDa in lysates from Lec2-DC-SIGN and Lec2-L-SIGN cells. Note that these species show a similar migration pattern to DC-SIGN and L-SIGN detected by Western blotting (Fig. 2B) and that

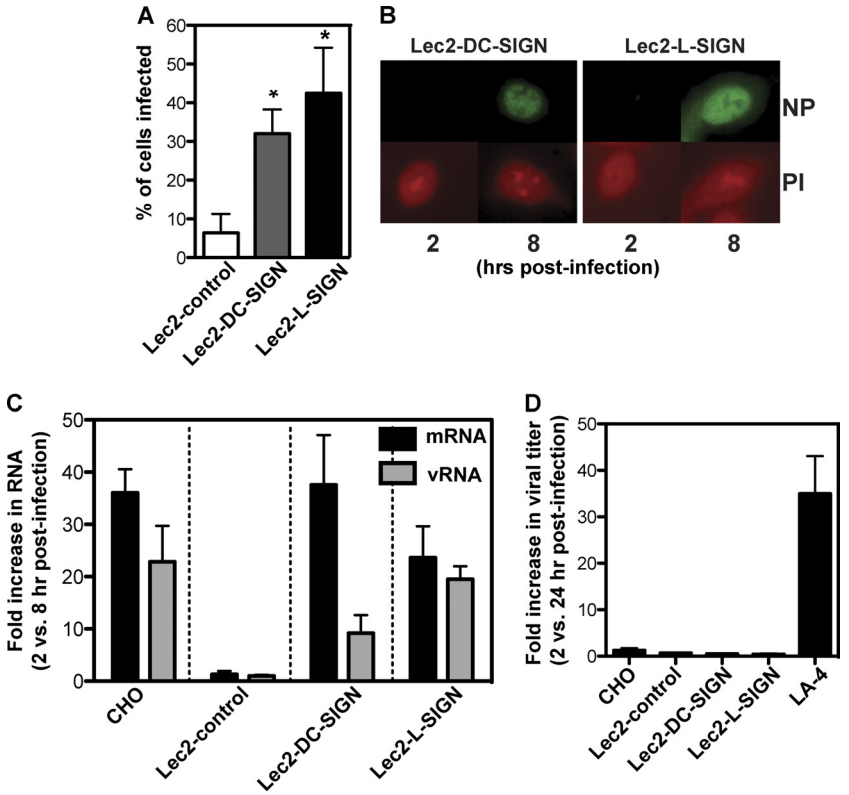

FIG. 5. DC-SIGN and L-SIGN expression in Lec2 cells enhances influenza virus infection. (A and B) Monolayers of Lec2-DC-SIGN, Lec2-L-SIGN, or Lec2-control cells were incubated with 10^7 PFU of influenza virus for 60 min at 37°C. Monolayers were washed, incubated 6 to 8 h and then fixed and stained by immunofluorescence for expression of newly synthesized viral NP. (A) Data represent the mean percent infection (± the SEM) from no less than four independent fields per chamber and are pooled from two separate experiments. (*, Significantly enhanced compared to Lec2-control cells [$P < 0.05$, one-way ANOVA].) (B) Lec2-DC-SIGN and Lec2-L-SIGN cells stained for viral NP (FITC) and double-stranded nucleic acids (propidium iodide [PI]) at 2 and 8 h postinfection (×100 magnification). (C) vRNA and mRNA levels of influenza A virus M gene in infected cells were determined by qRT-PCR. The data represent the mean fold increase (± the SEM) in vRNA and mRNA copy number between 2 and 8 h for each cell line. Assays were performed in triplicate and are representative of two independent experiments. (D) Enhanced infection of Lec2-DC-SIGN and Lec2-L-SIGN does not result in amplification and release of infectious virus. Cell monolayers were infected in chamber slides as described above. Cell supernatants were collected at 2 and 24 h postinfection, and titers of the infectious virus were determined by standard plaque assay. The data show the mean fold increase in virus titer (PFU/ml) between 2 and 24 h (± the SEM). Assays were performed in triplicate.

VOBPA failed to detect species of this molecular size in Lec2-control cells. Binding of virus to the 49- to 55-kDa species, but not to other bands detected, was abrogated in the presence of EDTA, a finding consistent with Ca^{2+}-dependent binding to C-type lectins. The identity of the other virus-binding bands detected in Lec2 cell lysates was not investigated further but may represent intracellular species, since binding of BJx109 to the surface of Lec2 CHO cells was negligible (Fig. 1B).

DC-SIGN and L-SIGN can mediate SA-independent entry of influenza A virus into cells. SA-deficient Lec2 cells expressing cell surface DC-SIGN or L-SIGN bind influenza virus in a Ca^{2+}-dependent manner. We next investigated the ability of DC-SIGN and L-SIGN to rescue the permissivity of Lec2 cells to infection by BJx109 virus. Compared to control Lec2 cells expressing intracellular OVA, expression of either DC-SIGN or L-SIGN was associated with a marked enhancement in susceptibility to infection by BJx109 (Fig. 5A). Virus-infected Lec2-DC-SIGN and Lec2-L-SIGN cells were identified using immunofluorescence to detect nuclear expression of viral NP at 8 h postinfection (Fig. 5B). After exposure of Lec2-DC-SIGN and Lec2-L-SIGN cells to BJx109, viral (v)RNA and messenger (m)RNA were markedly upregulated between 2 and 8 h postinfection, a result indicative of virus entry, genomic replication, and transcription (Fig. 5C). Consistent with immunofluorescence data (Fig. 1C and 5A), vRNA and mRNA were also upregulated in control CHO cells, but not in Lec2-OVA cells. These findings indicate that the poor ability of influenza virus to infect Lec2 cells does not represent a defect in the intrinsic ability of Lec2 cells to support virus replication but rather that Lec2 cells lack appropriate cell surface receptors for virus entry. The levels of infectious virus in supernatants from CHO, Lec2-DC-SIGN, or Lec2-L-SIGN cells exposed to BJx109 did not increase between 2 and 24 h postinfection (Fig. 5D), indicating that infectious progeny virus were not released from infected cells.

To formally address the role of cell surface SA and mannose-specific C-type lectins in the enhanced susceptibility of Lec2 transfectants to virus infection, cells were either (i) treated with bacterial sialidase prior to infection or (ii) incubated with mannan, a multivalent inhibitor of DC-SIGN and

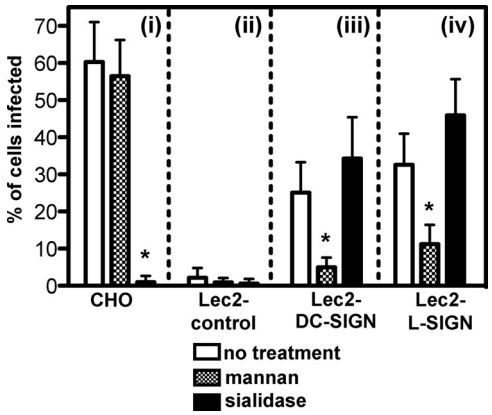

FIG. 6. Infection of Lec2-DC-SIGN and Lec2-L-SIGN cells by influenza virus infection is blocked by mannan but not by treatment of cells with bacterial sialidase. Monolayers of (i) parental CHO Pro 5 cells, (ii) Lec2-control, (iii) Lec2-DC-SIGN, or (iv) Lec2-L-SIGN cells in eight-well chamber slides were treated with either 10 mg of mannan/ml at 37°C for 60 min or 200 mU of bacterial sialidase/ml or were incubated with medium alone (no treatment) before infection with 10^7 PFU of BJx109. The percentage of infected cells was determined by using immunofluorescence at 6 to 8 h postinfection. The data represent the mean percent infection (± the standard deviation) from no less than four independent fields per chamber and are pooled from three independent experiments. (*, Significantly reduced compared to no treatment controls [$P < 0.05$, one-way ANOVA].)

L-SIGN, prior to and during infection with BJx109. As seen in Fig. 6i, BJx109 infected the parental cell line CHO Pro5 to high levels, and treatment with bacterial sialidase reduced infection to <5%, whereas the addition of mannan did not alter susceptibility to infection, consistent with SA-dependent virus entry. Lec2-control cells were largely resistant to infection (Fig. 6ii), and expression of either DC-SIGN (Fig. 6iii) or L-SIGN (Fig. 6iv) restored susceptibility to infection. In contrast to CHO Pro5 cells, inclusion of 10 mg of mannan/ml led to a marked reduction in susceptibility of Lec2-DC-SIGN and Lec2-L-SIGN to infection by BJx109.

Lec2 cells have markedly reduced levels of cell surface SA due to a mutation in the CMP-SA transporter; however, it is possible that this mutation may allow partial activity (36), enabling low-level SA expression. Therefore, it was important to determine any possible contribution of cell surface SA to the enhanced susceptibility of Lec2-DC-SIGN and Lec2-L-SIGN to virus infection. In contrast to CHO Pro5 cells (Fig. 6i), treatment of Lec2-DC-SIGN or Lec2-L-SIGN cells with bacterial sialidase did not reduce susceptibility to infection by BJx109 and was, in fact, generally associated with a modest enhancement in infection. Data are shown for cells treated with *Vibrio cholerae* (type III) sialidase, and similar results were obtained with an equivalent concentration of sialidase from either *Clostridium perfringens* or *Arthrobacter ureafaciens* (data not shown). Together, these data indicate that DC-SIGN- and L-SIGN-mediated infection of Lec2 cells can occur via lectin-mediated interactions in the absence of cell surface SA.

Glycosylation of the viral HA influences ability of influenza viruses to infect Lec2 cells expressing either DC-SIGN or L-SIGN. DC-SIGN and L-SIGN recognize mannose-rich oligosaccharides (21, 40), and influenza viruses are known to differ markedly in the glycosylation of HA and NA glycoproteins. In particular, virus strains differ in the degree of glycosylation on the globular head of HA (56), and this has been shown to modulate sensitivity to soluble C-type lectins (50, 51) and the MMR (49, 73). BJx109 (H3N2) has four potential sites of N-linked glycosylation on the globular head of the HA compared to PR8 (H1N1), which has none. BJx109 and PR8 bear three and two N glycosylation sites on the NA head, respectively (predictions were made by sequence analysis with the NetNGyc 1.0 Server). BJx109 virus is a high-yielding reassortant of PR8 with A/Beijing/353/89 (H3N2), bearing the H3N2 glycoproteins. ELISA wells coated with increasing concentrations of purified BJx109 and PR8 were probed with ConA, a plant lectin that binds high-mannose and hybrid-type oligosaccharides. BJx109 bound ConA more efficiently than PR8, and binding was inhibited in the presence of mannan (Fig. 7A). MAb 165, a carbohydrate-specific MAb that recognizes the cross-reactive host antigen common to all egg-grown influenza viruses (50), was used to confirm equivalent coating levels of virus (data not shown). Periodate treatment of BJx109 or PR8 to oxidize carbohydrate moieties expressed on HA/NA also abrogated ConA binding (Fig. 7A). Together, these data demonstrate that BJx109 expresses high levels of high-mannose and hybrid glycans, whereas PR8 does not.

To gain insight into the relative importance of HA and NA as ligands for DC-SIGN/L-SIGN, we utilized additional reassortant viruses engineered by reverse genetics (RG). Thus, either the HA or the NA of Beij/89 was inserted into the seven-gene backbone of PR8 by RG to generate RG-PR8-Beij/89 HA or RG-PR8-Beij/89 NA, respectively. In these experiments, PR8 was also rederived by RG (RG-PR8), as well as a virus bearing internal components of PR8 with Beij/89 HA and NA (RG-PR8-Beij/89 HA/NA). As seen in Fig. 7B, all RG viruses infected CHO Pro5 cells to equivalent levels (panel i) and were poor in their ability to infect Lec2-control cells (panel ii). RG-PR8-Beij/89 HA/NA infected Lec2-DC-SIGN (panel iii) and Lec2-L-SIGN cells (panel iv) to high levels, whereas RG-PR8 was particularly poor in its ability to infect Lec2 cells expressing either C-type lectin. Of interest, the RG virus bearing the Beij/89 HA (RG-PR8-Beij/89 HA) infected Lec2-DC-SIGN and Lec2-L-SIGN cells to levels equivalent to that of RG-PR8-Beij/89 HA/NA. In contrast, RG-PR8-Beij/89 NA infected Lec2 cells expressing either human C-type lectin very poorly. Together, these data indicate that expression of the HA glycoprotein of Beij/89 is associated with efficient infection by Lec2 cells expressing either DC-SIGN or L-SIGN.

The PR8 (H1N1) virus was particularly poor in its ability to infect Lec2 cells expressing DC-SIGN or L-SIGN, likely due to the low levels of glycosylation on the viral HA. In contrast, viruses bearing the highly glycosylated HA of Beij/89 (H3) infected cells efficiently. To confirm that the poor ability of PR8 to infect transfected cells was not a general feature of H1 subtype viruses, we used two additional virus strains, New Cal/99 and Sol Is/06, both of which bear four potential glycosylation sites on the head of HA. As seen in Fig. 7C, both viruses infected parental CHO Pro5 cells to equivalent levels (panel i) and were poor in their ability to infect Lec2-OVA cells (panel ii), although Sol Is/06 was somewhat more efficient than New Cal/99. In contrast, Lec2-DC-SIGN (panel iii) and Lec2-L-SIGN cells (panel iv) were both susceptible to infec-

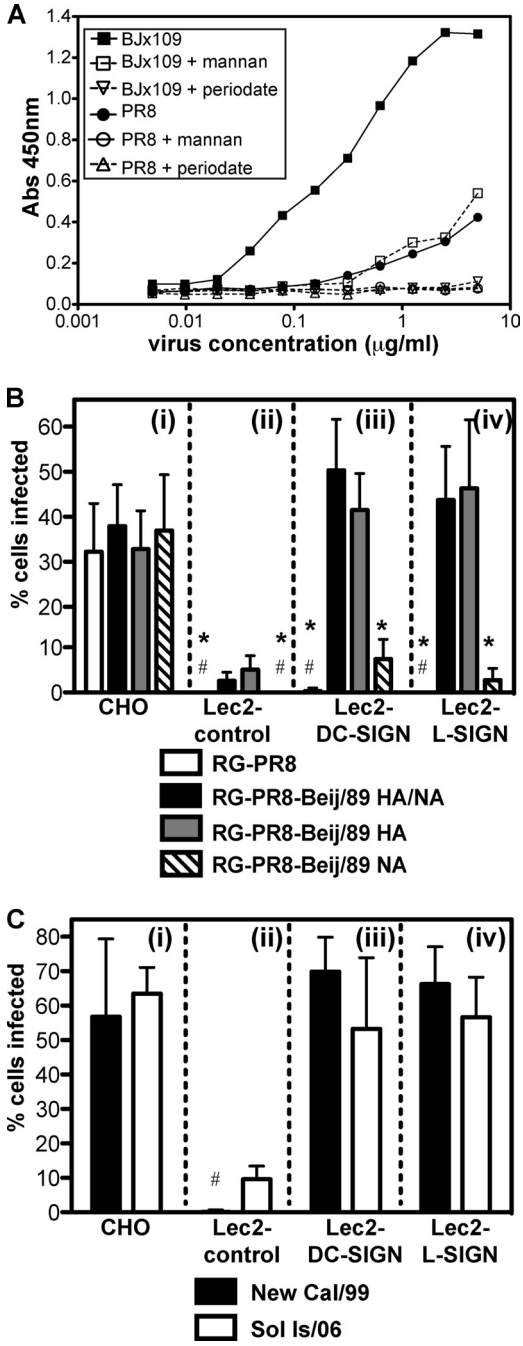

FIG. 7. Influenza viruses differ in their ability to infect Lec2-DC-SIGN and Lec2-L-SIGN cells. (A) Virus strains BJx109 and PR8 were compared for their ability to bind ConA by ELISA. Biotin-labeled ConA was added to wells coated with increasing concentrations of BJx109 or PR8 in buffer alone or buffer supplemented with 5 mg of mannan/ml (+ mannan). Wells coated with BJx109 or PR8 were also treated with 0.011 M sodium *meta*-periodate (+ periodate) for 20 min prior to the addition of ConA. Equivalent coating levels of BJx109 and PR8 were confirmed using MAb 165, which binds to the host-derived carbohydrate antigen characteristic of egg-grown influenza viruses (data not shown). MAbs directed to HA of BJx109 or PR8 confirmed that levels of virus were similar following treatment with periodate (data not shown). (B) Monolayers of parental CHO Pro5 (i), Lec2-control (ii), Lec2-DC-SIGN (iii), or Lec2-L-SIGN (iv) cells were infected with 5×10^6 PFU of RG-PR8, RG-PR8-Beij/89 HA/NA, RG-PR8-Beij/89 HA, or RG-PR8-Beij/89 NA. (C) Monolayers of parental CHO Pro5 (i), Lec2-control (ii), Lec2-DC-SIGN (iii), or Lec2-L-SIGN (iv) cells were infected with 3×10^6 PFU of either New Cal/99 or Sol Is/06. For panels B and C, the percentage of infected cells was determined by using immunofluorescence at 6 to 8 h postinfection as described above. The data represent the mean percent infection (± the standard deviation) from no fewer than four independent fields per chamber and are pooled from two independent experiments. (#, <1% of cells infected; *, significantly reduced compared to both RG-PR8-Beij/89 HA/NA and RG-PR8-Beij/89 HA [$P < 0.05$, one-way ANOVA].)

tion by either New Cal/99 or Sol Is/06 viruses. Thus, the poor ability of PR8 to infect Lec2 cells via DC-SIGN or L-SIGN is not a general property of H1N1 subtype viruses. More likely it relates to the low levels of mannose-rich glycans expressed by this virus (Fig. 7A).

DISCUSSION

Virus attachment and entry into host cells can be a multistep process involving sequential recognition of multiple receptors and cofactors. The present study has defined a system in which SA-independent interactions between influenza virus and putative cell surface receptors can be investigated. SA-deficient Lec2 cells did not bind efficiently to influenza A virus (Fig. 1B) and were resistant to infection (Fig. 1C). To our knowledge, this is the first description of a mammalian epithelial cell line that influenza virus does not bind to and/or infect efficiently. A related mutant CHO cell line (Lec1), deficient in N-linked glycans due to lack of GnT1 glycosyltransferase (61, 62), was largely resistant to infection by A/WSN/33 (H1N1) and A/Udorn/307/72 (H3N2) (12), although virus binding was unaffected presumably due to expression of SA on O-linked glycoproteins and glycosphingolipids.

Previous studies implicating C-type lectins (e.g., MMR and MGL) in infectious entry of influenza virus into murine Mϕ have been informative but not definitive (49, 73). We hypothesized that expression of C-type lectins by Lec2 cells would allow virus attachment/entry to be assessed without the added complexity associated with HA-mediated recognition of cell surface SA. In previous studies, transfection of Lec2 CHO cells led to abundant biosynthesis of MMR, but recognition of mannose-specific ligands was compromised, indicating a critical role for terminal sialylation in ligand binding (65). In contrast, human DC-SIGN and L-SIGN expressed by Lec2 CHO cells mediated Ca^{2+}-dependent binding to mannan (Fig. 3) and to virus (Fig. 4). DC-SIGN and L-SIGN express a single N-linked glycosylation site near the neck region of each molecule (GenBank accession numbers M98457 and AF245219), and removal of this site increased the multimerization and lectin function of DC-SIGN (55). Consistent with our data, recombinant DC-SIGN and L-SIGN expressed in *Escherichia coli* retained the ability to bind mannose-rich glycans (40).

Expression of DC-SIGN and L-SIGN restored the capacity of Lec2 cells to support influenza virus infection (Fig. 5A). To our knowledge, this is the first demonstration that L-SIGN can play a role in influenza virus attachment and entry. Wang et al. implicated DC-SIGN in promoting H5N1 virus infection as a capture/attachment molecule rather than a virus entry receptor

(76). H5N1-pseudotyped particles were captured by DC-SIGN expressed by B-THP-1 (human Raji B cells) and THP-1 (human monocytic cells) to enhance infection in *cis* and to transfer virus to permissive cells in *trans*. However, desialylation was associated with reduced virus infection, an observation consistent with the ability of DC-SIGN to facilitate infection in *cis* via SA-expressing receptors (76). Sialylated species have been reported as absent (44) or present in small amounts (36) on Lec2 cells, and we observed low levels of sialidase-sensitive binding of MAA to Lec2 cells (Fig. 1). Treatment of DC-SIGN-Lec2 and L-SIGN-Lec2 cells with bacterial sialidase confirmed that attachment and entry of virus can occur independently of cell surface SA.

Influenza virus HA/NA express a mixture of high-mannose and complex oligosaccharides, including glycans bearing terminal galactose, mannose, and fucose residues (4, 13, 51, 77). ConA binding confirmed BJx109 expressed high levels of mannose-rich glycans compared to PR8 (Fig. 7A), and BJx109 infected Lec2-DC-SIGN and Lec2-L-SIGN efficiently, whereas PR8 did not (Fig. 7B). Moreover, the ability of BJx109 to infect Lec2-DC-SIGN and Lec2-L-SIGN was blocked by mannan (Fig. 6). Together, these data indicate that differences in glycosylation are likely to modulate recognition and/or internalization of influenza virus by DC-SIGN/L-SIGN. Site-directed mutagenesis has defined particular oligosaccharides expressed by WNV (16) and SARS-CoV (29) that promote interaction with DC-SIGN/L-SIGN. Addition of specific sites of N glycosylation to PR8 HA and characterization of the oligosaccharide composition of HA of different virus strains will provide important information regarding specific ligands for DC-SIGN/L-SIGN expressed by influenza viruses.

The principal targets for influenza virus infection in humans are cells of the upper and lower airways. L-SIGN is expressed by bronchiolar epithelial cells, type II alveolar cells, endothelial cells of the lung, and subsets of lung stem/progenitor cells (10, 30). Influenza virus infection of epithelial cells and endothelial cells generally results in productive replication and virus amplification (8, 66). Therefore, we postulate that recognition of influenza virus by L-SIGN on cells in the lung may contribute to sustained replication and spread of virus *in vivo*. In contrast, DC-SIGN is expressed on human alveolar Mϕ (60) and subpopulations of lung DCs, including interstitial-type DCs (68, 74), and expression is modulated during infection and inflammation. It has been reported that human Mϕ do (11, 45) and do not (52) support productive virus replication. The outcomes of virus infection described herein (i.e., nonproductive replication) in CHO/CHO Lec2 cells are likely to be very different to those in primary cells, and further studies are required to elucidate responses of human airway Mϕ/DC subsets to influenza virus. DC-SIGN and L-SIGN recognize mannose-rich glycans but only DC-SIGN displays affinity for fucosylated oligosaccharides (27). Moreover, binding of DC-SIGN by distinct pathogens can lead to inhibition or promotion of particular T-cell responses (5, 57), which may relate to the distinct signaling pathways induced by mannose and fucose-expressing pathogens (26). In light of such findings, it will be of particular interest to determine the composition of glycans expressed by different influenza viruses and how these impact on intracellular signaling and cytokine production by human Mϕ/DCs.

DC-SIGN has been implicated as a direct route for infection of cells by dengue virus (69), cytomegalovirus (CMV) (28), Ebola virus (1), and WNV (16) and can promote infection of permissive cells in *trans* by Ebola virus (1), hepatitis C virus (15), and CMV (28). L-SIGN has been reported to capture and transfer hepatitis C pseudovirus to human hepatocytes (15), as well as promoting infection in *cis* by WNV (16), dengue virus (69), CMV (28), and Ebola virus (1). Of interest, DC-SIGN was shown to mediate endocytosis, receptor recycling, and the release of ligand at endosomal pH, whereas L-SIGN did not (27). However, L-SIGN-mediated uptake and trafficking of ligand into endosomal compartments (75), as well as the uptake and degradation of SARS-CoV, have been reported (9). Our data suggest that L-SIGN-mediated delivery of influenza virus into the endosomal pathway facilitates fusion of viral and endosomal membranes in the late endosome, resulting in commencement of viral replication. Moreover, efficient infection of SA-deficient DC-SIGN-Lec2 and L-SIGN-Lec2 cells suggest that HA-SA interactions are not always required for efficient pH-induced fusion (18).

Expression of putative receptors in Lec2 cells removed the confounding factor of multiple low-affinity interactions between influenza virus HA and cell surface SA. While SA was long considered to be the sole receptor for influenza virus, studies have shown that desialylated mammalian cells can support virus infection (64, 70). In addition to DC-SIGN and L-SIGN, other cell surface lectins might also mediate SA-independent binding and/or entry. Rapoport et al. demonstrated that oligosaccharide probes showed different patterns of binding to MDCK and Vero cells (48), which is consistent with the notion that cell surface galectins and/or mannose-binding lectins could potentially bind virus. Multiple pathways are likely to exist for influenza virus entry into cells (reviewed by Nicholls et al. [43]), and understanding the mechanisms of SA-independent virus entry may have relevance to development of potential treatments for influenza, including inhaled sialidases (71). As demonstrated here, virus attachment and entry can occur independently of SA; however, efficiency might be markedly enhanced if virus were concentrated at the cell surface by low-affinity interactions with SA. Such a multistep model would allow virus to "browse" the cell surface (7) before recognition by secondary receptors such as DC-SIGN or L-SIGN for subsequent entry.

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